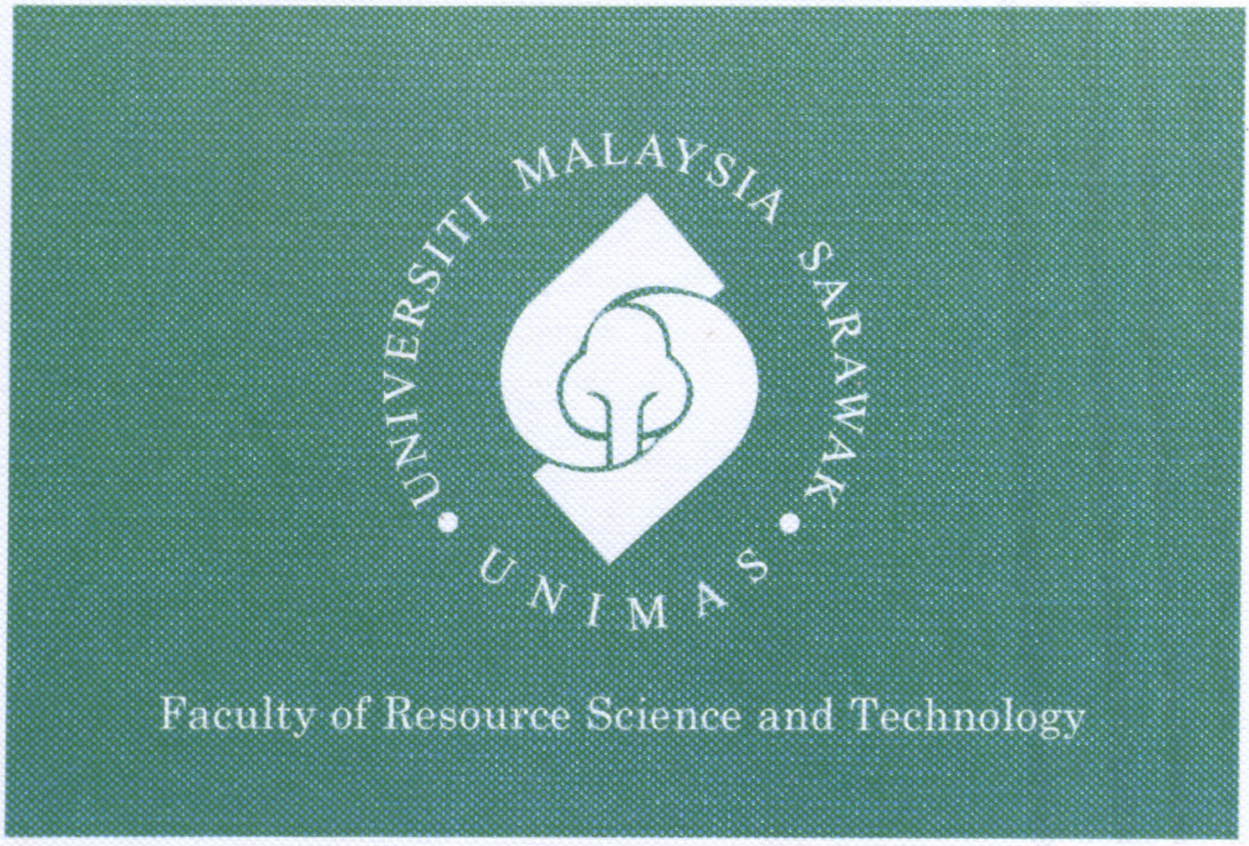

PHYLOGENETIC RELATIONSHIP ON GENUS *LEPTOBRACHIUM* (AMPHIBIA: ANURA: MEGOPHRYIDAE) IN SARAWAK

Norhasimah Binti Jakariah
(31890)

Bachelor of Science with Honours
(Animal Resource Science and Management)
2014

UNIVERSITI MALAYSIA SARAWAK

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Final Year Project Report ☐

Masters ☐

PhD ☐

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PHYLOGENETIC RELATIONSHIP OF GENUS *LEPTOBRACHIUM* (AMPHIBIA: ANURA: MEGOPHRYIDAE) IN SARAWAK

NORHASIMAH BINTI JAKARIAH (31890)

This project is submitted in partial fulfillment of the requirement for the degree of a Bachelor of Science with Honours
(Animal Resource Science and Management)

Department of Zoology
Faculty of Resource Science and Technology
UNIVERSITY MALAYSIA SARAWAK
2014

DECLARATION

I hereby declare that no portion of the work referred to this dissertation has been submitted in support of an application for another degree or qualification to this or any other university or institute of higher learning.

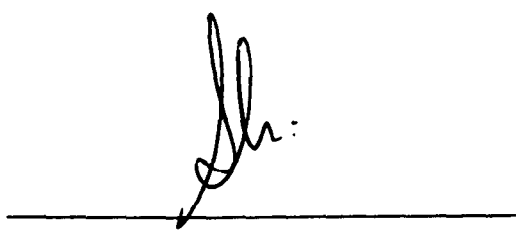

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Bp	Base Pair
16S rRNA	16s Ribosomal RNA
1FL	First Finger Length
3FL	Third Finger Length
CIA	Chloroform – Isoamyl Alcohol
CTAB	Cetyltrimethylammonium Bromide
DNA	Dinucleotide Acid
EDTA	Ethylene-Diamine-Tetra-acetic Acid
EL	Eye Length
FL	Foot Length
HAL	Hand Length
HL	Head Length
HLL	Hindlimb Length
HW	Head Width Upper arm Length

ICD	Intercanthal Distance (Distance Between Eyes)
IMTL	Inner Metatarsal Tubercle Length
IND	Length Internarial Distance (Distance Between Nostril)
IPTL	Inner Palmar Tubercle Length
LAL	Lower Arm and Hand Length
MEGA	Molecular Evolutionary Genetic Analysis
MgCL2	Magnesium Chloride
ML	Maximum Likelihood
MP	Maximum Parsimony
mtDNA	Mitohcondrial Deoxyribonucleic Acid
NaCl	Sodium Chloride
N-EL	Nostril To Eye Distance
NJ	Neighbour Joining
OPTL	Outer Palmar Tubercle Length
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid

SL	Snout Length
S-NL	Snout to Nostril Length
SVL	Snout-to-Vent Length
TAE	Tris-Acetate- EDTA
TD	Tympanum Diameter
TEL	Tympanic- Eye Length
TL	Tibia Length

LIST OF TABLES

LIST OF FIGURE

Phylogenetic Relationship of Genus *Leptobrachium* (Amphibia: Anura: Megophridae) in Sarawak

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ABSTRACT

Genus *Leptobrachium* is one of genera within family Megophridae found in Sarawak. It consists of cryptic species that are difficult to distinguish. Thus, phylogenetic relationship of genus *Leptobrachium* was studied by using mitochondrial ribosomal of 16S rRNA. Tissue sample were collected in four localities within Sarawak namely Kubah National Park, Matang Wildlife Centre, Mt. Gading National Park, and Mt. Santubong, Sarawak by using forest transects method and quadrats method. Tissues were extracted using molecular technique approach such as DNA extraction, PCR amplification, and sequencing. Phylogenetic relationships were inferred using primer pair of mitochondrial ribosomal 16S rRNA gene. A total of 1464bp of 16S rRNA from 10 samples were analyzed using Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) method. For this study, monophyletic group were formed consist of all species within the genus *Leptobrachium* with respect to outgroup. It is proven that 16S rRNA gene was useful as gentic marker to elucidate phylogenetic study within genus level. The study of phylogenetic relationship of genus *Leptobrachium* able to infer the divergence and lineage of species within respected genus thus contribute to provide future reference at local level.

Keywords: Phylogenetic, 16S rNA, *Leptobrachium,* Maximum-likelihood, Monophyletic

ABSTRAK

Genus Leptobrachium merupakan salah satu genera di bawah keluarga Megophridae yang terdapat di Sarawak. Ia terdiri daripada species kriptik yang sukar untuk dikenal pasti. Oleh itu, hubungan filogenetik genus Leptobrachium telah dikaji dengan menggunakan ribosom mitokondria daripada 16S rRNA. Sampel tisu telah didapati daripada empat kawasan di Sarawak iaitu Taman Negara Kubah, Pusat Hidupan Liar Matang, Taman Negara Gading, dan Gunung Santubong, Sarawak dengan menggunakan kaedah transek hutan dan kaedah kuadrat. Tisu di extrak menggunakan pendekatan teknik molekul seperti pengekstrakan DNA, PCR, dan sekuensing. Hubungan filogenetik telah disimpulkan menggunakan pasangan primer asas daripada mitokondria ribosom 16S rRNA gen. Sebanyak 1464bp daripada 16S rRNA dari 10 sampel telah dianalisis dengan menggunakan kaedah Neighbor - Menyertai (NJ), kaedah Maksimum Parsimony (MP) dan kaedah Maximum Likelihood (ML). Untuk kajian ini, kumpulan monofiletik telah dibentuk terdiri daripada semua spesies dalam genus Leptobrachium berkenaan dengan keluarga luar. Ia membuktikan bahawa 16S rRNA gen berguna sebagai penanda genetik untuk menjelaskan kajian filogenetik dalam tahap genus. Kajian hubungan filogenetik genus Leptobrachium dapat membuat kesimpulan pencaran dan keturunan spesies dalam genus dengan itu menyumbang dalam menyediakan rujukan masa depan di pada peringkat tempatan.

Kata kunci: Filogentik, 16S rRNA, Leptobrachium, Maximum- likelihood, Monofiletik

CHAPTER 1

INTRODUCTION

1.1 Background of study

Frogs are among the most diverse vertebrates in the world as they appear very abundance throughout the world. Currently, 6350 living species of Anuran were recognized (Frost, 2014). Frogs are widely distributed and continuously to grow especially in the island of Borneo. It is reported that in Borneo there are rich in biodiversity especially in the group of herpetofauna (Das, 2006). In Borneo, it is reported that 154 species of frog have been recorded whereas 89 species are classified as endemic to Borneo and expected the number of species recognized will continuously to grow (Inger and Stuebing, 2009). The abundance of frogs in Borneo is mainly due to the ecological suitability of certain frogs species. There are 6 families of frog recorded in Borneo which are Bombinatoridae, Bufonidae, Megophyridae, Microhylidae, Ranidae, Rhacophoridae (Inger and Stuebing, 2005).

Megophyridae is one of the families consist of many cryptic species and most are endemic to Borneo. It is known as Bornean litter frog consists of four genera within family with total of 22 species recorded in Borneo (Inger and Stuebing, 2005). Current status of total species was increased to total of 25 species (Frost, 2014). Among 22 species, 15 species were recorded from Sarawak and four of species are endemic to Sarawak Inger and Stuebing (2005). With current status, number of endemic species was also increase to five species (Frost, 2014).

Four genera were recognized within Bornean Megophryidae including genus *Leptobrachella*, genus *Leptobrachium*, genus *Leptolalax*, and genus *Megophrys*. Species of genus *Leptobrachium* recorded in Sarawak were *L. montanum* Fischer, 1885, *L. abbotti* Cochran, 1926, *L. hendricksoni* Taylor, 1962, and *L. nigrops* Berry and Hendrickson, 1963 (Inger and Stuebing, 2005). This includes three new species described which are *L. ingeri* Hamidy, Matsui, Nishikawa, and Belabut, 2012, *L. kanowitense* Hamidy, Matsui, Nishikawa, and Belabut, 2012 and *L kantonishikawai* Hamidy and Matsui, 2014 (Frost, 2014).

1.2 Problem Statement

The study on phylogeny of Asian spade foot of genus *Leptobrachium* is relatively poor and was not well understood (Matsui, 2010). Taxonomic problem occur in *Leptobrachium* span from supraspecific to the species level (Matsui, 2010). Hence, crypticity occurrence among the species within genus *Leptobrachium* itself has contributed to this problem. Recent molecular study had found that both *L. montanum* and *L. abbotti* contain several cryptic species where there are morphological and genetic discordance occurs within this both species in north western of Borneo (Hamidy *et al.*, 2011) and later study come out with description of new species of *L. kantonishikawai* (Hamidy and Matsui., 2014). It is similar to other subgenus of Bornean *Leptobrachium* which is *L. nigrops* where study by Hamidy *et al.* (2012) had detected crypticity within these taxa at population level and distinguished *L.nigrops* from coastal Sarawak as *L.ingeri* and from inland Sarawak as *L.kanowitense*. There are many more cryptic species await description from Borneo (Hamidy *et al.*, 2011; Hamidy *et al.*, 2012). Study on molecular alone especially within cryptic species might give less rigid evidence to clear the status on phylogeny structure of genus *Leptobrachium*.

Morphological analysis data based on multivariate analysis of discriminant function analysis (DFA) were also included in this study.

In this project the study were focused on genus *Leptobrachium* Tschudi of Bornean Megophyridae within Sarawak. The aim of this study was to construct phylogenetic relationship of genus *Leptobrachium* in Sarawak by using mitochondrial ribosomal of 16S gene sequence. Based on previous study, it is stated that mitochondrial ribosomal of 16S gene is a good molecular marker as it fulfil the requirements for a universal DNA barcoding marker in amphibian (Vences *et al.*, 2005). This is also to promote a comparative study on molecular and morphometric analysis to validate the taxonomic status of genus *Leptobrachium* to assist molecular phylogeny studies especially within cryptic species. This study also importance as it could inferred the information on phylogenetic relationship of genus *Leptobrachium* such as their divergence and lineage, and also able to assist on the identification of species of genus *Leptobrachium* in Sarawak based on molecular approaches with aid of data from morphometric analysis. This study would contribute to provide future reference especially at local level.

1.3 Objectives of study

I. To construct phylogenetic tree of four species in genus *Leptobrachium* of family Megophyridae in Sarawak by using mitochondrial ribosomal of partial 16S gene sequence.

II. To determine whether mitochondrial ribosomal 16S gene is a good genetic marker to infer phylogenetic relationship of species within genus *Leptobrachium*.

CHAPTER 2

LITERATURE REVIEW

2.1 Systematics, Habitat and Distribution

Megophyridae is one of the families that found in Sarawak. The name of Megophyridae is defined based on Greek words mean large eyebrow. The family of Megophyridae is distributed to southeast continental Asia, from Pakistan to western China, east to the Philippines and the Sunda Island (Frost, 2014). Family of Megophryidae or common named litter frog is classified under the order of anuran. Megophryidae were classified as sister taxon of Pelobates based on adult characters (Pugener, 2003) and subfamily of Pelobatidae (Haas, 2003). However it is later revised by frost *et al.* (2006) through molecular study had provided taxonomic history and a partial phylogenetic analysis to reject the subfamilies of Megophryidae and separate the rank as independent family.

Overall, a total of 181 species of Megophryidae from nine genera were recorded from Pakistan and western China east to the Philippines and the Greater Sunda Islands (Frost, 2014). The species of Megophryidae often found seen along the river bank, sometimes wander over the leaf litter of forest floor and near the rocky stream of secondary forest at lowland (Inger and Stuebing, 2005). The species also recorded to inhabit highland of 1000m above the sea level.

In Borneo, there are total of 22 species were and within 22 species, 17 species can be found in Sarawak (Inger and Stuebing, 2005; Hamidy and Matsui, 2012) where five of

species were endemic to Sarawak. However, recent study by Hamidy and Matsui (2012) had provided three descriptions on new species of genus *Leptobrachium* which make total species found in Borneo increased to 25 species. Four genus of Megophridae distributed in Borneo are *Leptobrachella, Leptobracium, Leptolalax* and *Megophrys* (Inger and Stuebing, 2005; Matsui, 2010; Frost, 2014). Species of *Leptobrachium* including new species known in Sarawak are as follows: *L. montanum* Fischer, 1885, *L. abbotti* Cochran, 1926, *L. hendricksoni* Taylor, 1962, *L. nigrops* Berry and Hendrickson, 1963 (Inger and Stuebing, 2005), *L. ingeri* Hamidy, Matsui, Nishikawa, and Belabut, 2012, *L. kanowitense* Hamidy, Matsui, Nishikawa, and Belabut, 2012, and *L kantonishikawai* Hamidy and Matsui, 2014 (Frost, 2014).

The megophryid genus *Leptobrachium* Tschudi, 1838 is considered to contain two subgenera *Vibrissaphora* Liu, 1945, and *Leptobrachium* in which the presence of with bearing spines on upper lip in adult males differentiate this two subgenera (Ohler *et al.*, 2004). Zheng *et al.* (2008) and Rao and Wilkinson (2008) had place *Vibrissaphora* within the genus *Leptobrachium* and neither group of researchers recognizes subgenera. 34 species within the genus *Leptobrachium* were known throughout Southern China and India to islands of the Sunda Shelf and the Philippines (Hamidy and Matsui, 2014; Frost, 2014). In Borneo alone there are total of 25 species were recorded (Hamidy and Matsui, 2012). Inger (1966) and Berry (1975) had applied the name of *L. hasseltii* to most of Southeast Asian population. It is clarified that *L. hasseltii* from Borneo was not conspecific with the Javanese population and thus applied the names *L. montanum* and *L. abbotti* to Bornean species (Inger *et al.*, 1995). Previous study by Hamidy *et al.* (2011) had stated that there are morphological and genetic discordance occurs in species, *L. montanum* and *L. abbotti* as crypticity were found within both species. They have only slightly difference in

morphology where it can be identified only in live specimens. At population level study by Hamidy and Matsui (2014) had also describe new species of *L. kantonishikawai* from Bario, Kelabit Highland of Sarawak as it is once confused as *L. abbotti.* The Bario population was known to have characteristics distinct from all other congeners.

Similar to other congeneric species, *L.nigrops* was once described from Southeast Asia and treated as *L. hasseltii* (Taylor, 1962). However, it is revised later by Berry and Hendrickson (1963), describe that Singapore as the type locality of *L. nigrops* and the populations of *L. hasseltii* from Singapore and Peninsular Malaysia were distinct species. It is known that population of *L. hasseltii* is restricted to Bali and Java, and other population of northeastern India to the Philippines was once treated as *L. hasseltii* was assigned to other named (Dubois and Ohler, 1998; Matsui, Nabhitabhata, and Panha, 1999; frost, 2014). There are high levels of genetic divergences among allopatric populations of *L. nigrops* (Brown *et al.,* 2009; Matsui *et al.,* 2010) where three clades of populations from Malay Peninsula, Belitung, and inland area of Borneo were recognized. Taxonomic relationship of *L. nigrops* were later evaluated based on molecular study by Hamidy and Matsui (2012) describe true population of *L. nigrops* were from Singapore and Malay Peninsula while populations from coastal area of Sarawak as *L. ingeri* and the population from the inland area of Sarawak as *L. kanowitense.*

L. hendricksoni is distinct in having black spots on its venter and an orange-colored upper (and sometimes whole) iris (Inger, 1966). Matsui *et al.* (2010) stated that *L. hendricksoni* in Peninsular Malaysia was paraphyletic with respect to Sumatran populations and populations from the northern part of Peninsular Malaysia are the sister group to the Sumatran populations and together form the sister group to the southern peninsular populations. Genetic diversity of *L. hendricksoni* was much smaller compare to *L. nigrops*, thus suggesting a unique evolutionary history (Matsui *et al.*, 2010). Taxonomic status on population of *L. hendricksoni* was not much to be known as they are rarely to be found.

2.2 Mitochondrial ribosomal 16S rRNA gene

In this study, the evolutionary relationship of genus *Leptobrachium* of Megophyridae in Sarawak was constructed by using mitochondrial ribosomal of 16S rRNA gene sequence. It fulfill the requirements for a universal DNA barcoding marker in amphibian by having this few characteristic such as sufficiently variable to unambiguously identify most species, highly conserved mitochondrial marker for the marker to be less variable within species, able to convey sufficient information on phylogenetic study and the sequence alignment will be possible among distantly related taxa (Vences *et al.*, 2005).

2.3 Morphometric

Morphometric is one of fundamental area of research involve quantitative description and analysis on variation of shapes of structures based on morphology of organisms. It is one of techniques that used in systematic study (Rohlf, 1990). Term of morphometric is taken from greek words with direct definition as 'morph' (form) and 'metrikos' (measure). Several method of morphometrics analysis was known to be widely used among biological investigators. This included multivariate morphometrics, coordinate morphometrics, boundary morphometrics and structural morphometrics (Lestrel, 2000). Morphometrics plays an important post-cladistic role in the analysis of trends and responses to evolutionary causes and constraints (Smith, 1990). A traditional morphometrics used in phylogenetics were commonly using data in term of size and shape variable (Smith, 1990). Commonly, a primary method used for traditional morphometric is multivariate statistics which applied for both animal and botanical.

2.3.1 Discriminant Function Analysis

Discriminant function analysis is a multivariate statistical analysis used to predict a categorical dependent variable by one or more continuous or binary independent variable. DFA aid to determine which continuous variables discriminate between two or more naturally occurring groups (Poulsen and French, 2004). Study by Multivariate analyses of variance using discriminant function able to interpret morphological differences between the two cryptic species in relation to factors that influence the morphology characters (Klimov, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collections

Field sampling of *Leptobrachium* species was conducted from study sites located within Sarawak namely Kubah National Park (N01°36' 19.3" E110°11'30.3"), UNIMAS east campus (01° 27'N; 110° 27'E), Mt. Gading National Park (N01°41' 49.5" E109°50'92.7"), and Mt. Santubong, Sarawak (N01°44'0" E110°20'0"). Samples were collected based on standard method of two types of line transect which are forest transect and stream transect, and forest floor quadrat method. The sampling started during night from hour 1930 until 2130 for transects and during the day from hour 0800 until 1200 for forest floor quadrat. All ecological data including snout-vent length and weight of specimen was recorded. Details of tissue used are shown in table 1. Tissue sample was taken from thigh muscle and put into cryovial containing 99% absolute ethanol for preservation and stored in 20°C freezer for long term storage. The voucher tissue samples of previous collection from other localities were also included in this study. The specimen collected was tagged and the cryovial tube was marked following the specimen tags. The specimens were preserved in 10% formalin and brought back to UNIMAS for further processing.

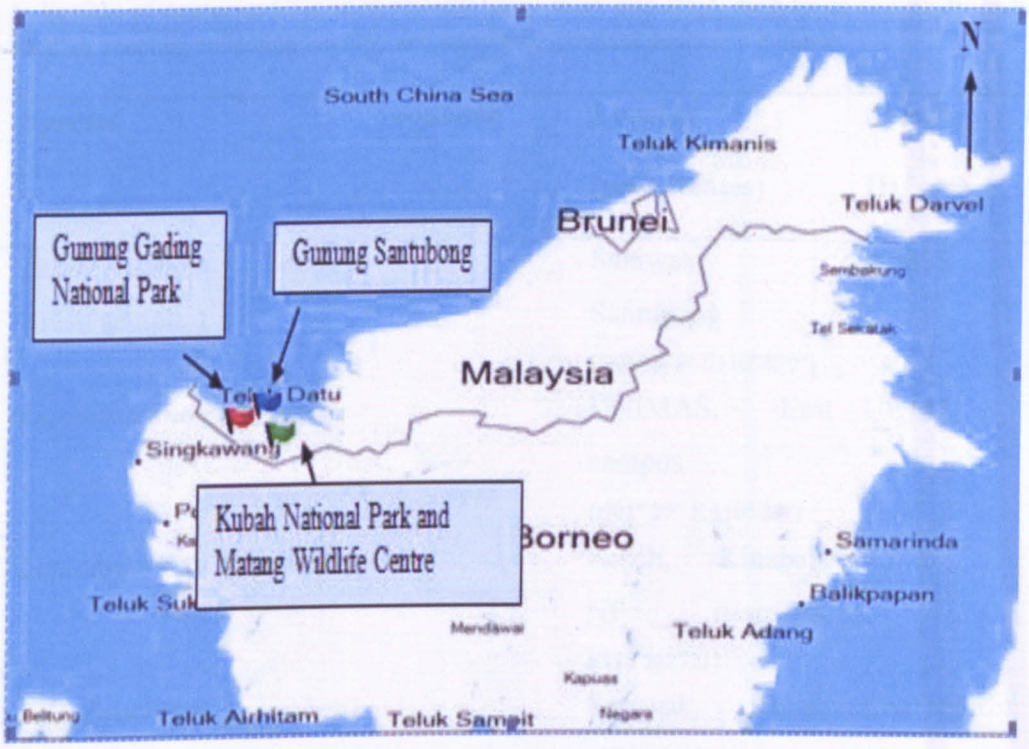

Figure 1: Map of study site within Sarawak (Source: map source)